

Cytoprotective effect of β -lapachone by inducing heme oxygenase-1 expression and AMP-activated protein kinase activation in human endothelial cells

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Abstract. – **OBJECTIVES:** AMP-activated protein kinase (AMPK) is suggested to exert cytoprotective and anti-inflammatory effects in endothelial cells, but the precise mechanisms are not fully understood. It has been reported that pharmacological activation of AMPK induces endothelial heme oxygenase-1 (HO-1) expression. β -Lapachone (BL), a well-known substrate of NAD(P)H:quinone oxidoreductase (NQO1), stimulates AMPK activation via NQO1 activation. Here we examined whether AMPK activation by BL would be linked to HO-1 expression in ECV304 endothelial cells and whether HO-1 expression could mediate the cytoprotective effect of BL.

MATERIALS AND METHODS: Endothelial cells were pre-incubated for 6 h with BL or 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) in the absence or presence of dicoumarol (DC), compound C (CC), or tin protoporphyrin-IX (SnPP), and then challenged with tumor necrosis factor- α (TNF- α) for 24 h. Cell viability was evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. AMPK phosphorylation and HO-1 expression were detected by Western blot analysis.

RESULTS: At non-cytotoxic concentrations, BL induced AMPK phosphorylation and HO-1 expression. AICAR, an AMPK activator, also induced HO-1 expression. In contrast, CC, an inhibitor of AMPK activation, and DC, an inhibitor of NQO1, prevented the increase in BL-induced HO-1 expression. Pretreatment with BL or AICAR reduced TNF- α -induced endothelial cell death. Cytoprotection by BL was almost completely abolished by CC and DC and partly by SnPP, a competitive inhibitor of HO-1.

CONCLUSIONS: Our results suggest that BL induces cytoprotective HO-1 expression in endothelial cells via AMPK activation, providing one of possible mechanisms by which BL can exert beneficial effects.

Key Words:

β -Lapachone, AMP-activated protein kinase, Heme oxygenase-1, Tumor necrosis factor- α , Endothelial cell.

Introduction

Endothelial cell injury leading to endothelial dysfunction has been regarded as the critical event in the pathogenesis of most cardiovascular diseases being associated with atherosclerosis, hypertension, coronary and peripheral artery dysfunction, chronic heart failure, diabetes mellitus, insulin resistance, and inflammation¹. Although the detailed mechanisms leading to endothelial cell injury remain incompletely understood, uncontrolled formation of reactive oxygen species (ROS) is known to induce such injury². A candidate factor in causing excessive ROS production in endothelial cells is tumor necrosis factor alpha-a (TNF- α), a pleiotropic pro-inflammatory cytokine. It had long been noticed that TNF- α could cause injury to endothelial cells resulting in endothelial dysfunction, but the underlying mechanisms have not been fully understood. ROS have been shown to serve as one of the key mediators involved in TNF- α -induced cellular injury³.

AMP-activated protein kinase (AMPK), an energy-sensing enzyme that regulates energy homeostasis and metabolic stress, is activated primarily by increase in cellular AMP-to-ATP ratio, which can occur in various conditions, such as glucose deprivation, heat shock, oxidative stress, and ischemia⁴. However, pharmacologically relevant molecules, such as 5-aminoimidazole-4-

carboxamide-1- β -D-ribofuranoside (AICAR), A769662, and metformin, are also capable of activating AMPK independently of changes in the AMP-to-ATP ratio⁵. Recent studies have demonstrated that AMPK activation has cytoprotective and anti-inflammatory effects in endothelial cells^{6,7}. However, the molecular mechanism(s) by which AMPK activation can exert these effects remains unclear. Interestingly, it has been demonstrated that AMPK activation stimulates the expression of the cytoprotective and anti-inflammatory heme oxygenase (HO)-1 in endothelial cells^{8,9}.

HO-1 is a rate-limiting enzyme in the degradation of free heme to produce free iron, carbon monoxide and biliverdin, which is further converted to bilirubin by biliverdin reductase¹⁰. Through these byproducts, HO-1 mitigates cellular injury by exerting antioxidant, cytoprotective, and anti-inflammatory effects. Indeed, numerous studies have been reported to prove the effect of HO-1 in the prevention of endothelial cell injury¹¹⁻¹⁴.

NAD(P)H:quinone oxidoreductase 1 (NQO1) catalyses the oxidation of NADH to NAD⁺ by various quinones¹⁵⁻¹⁷. Several activators and substrates of NQO1 have been identified. One of the well-known NQO1 substrates is b-lapachone (BL) isolated from the bark of the *Lapacho* tree¹⁸. Recent studies have indicated that BL stimulates AMPK activation by increasing NAD⁺-to-NADH ratio *via* NQO1 activation; so being considered as a novel AMPK activator¹⁹.

The previous findings demonstrating that BL stimulates AMPK activation by enhancing NQO1 activity^{19,20} and AMPK activation induces HO-1 expression^{8,9} prompted us to examine whether AMPK activation by BL would be linked to endothelial HO-1 expression and, if so, whether HO-1 could mediate the cytoprotective effects of BL in ECV304 endothelial cells.

Materials and Methods

Reagents and Antibodies

BL, compound C (CC), AICAR, tin protoporphyrin-IX (SnPP), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), dicoumarol (DC), *N*-acetylcysteine (NAC), propidium iodide (PI), and Dulbecco's modified Eagle's medium (DMEM) were obtained from Sigma-Aldrich (St. Louis, MO, USA). TNF- α was purchased from BD (Becton-Dickinson & Co, Franklin Lakes, NJ, USA). A polyclonal HO-1 antibody was from StressGen Biotechnologies

(Victoria, Canada), antibodies directed against AMPK, phospho (P)-AMPK, and β -actin were from Cell Signaling Technology (Beverly, MA, USA), and secondary antibodies was from Santa Cruz Biotechnologies (Santa Cruz, CA, USA).

Cell Culture

ECV304 cells derived from normal human vascular endothelial cells were obtained from the ATCC: American Type Culture Collection (Manassas, VA, USA) and cultured in plastic flasks in DMEM, containing: 10% fetal bovine serum (FBS), 1% *L*-glutamine (2 mM), 1% penicillin (10,000 IU/ml) and 1% streptomycin (10,000 μ g/ml). Exponentially growing cell cultures were maintained at 37°C, in a humidified atmosphere containing 5% CO₂ and 95% O₂. Confluent cultures of ECV304 endothelial cells were pre-incubated for 24 h in DMEM containing 1% serum before experiments.

Cell Viability Assay

Cell viability was determined by a modified MTT reduction assay. MTT is a pale yellow substance that is reduced by living cells to yield a dark blue formazan product. This process requires active mitochondria, and even fresh dead cells do not reduce significant amounts of MTT. ECV304 endothelial cells were cultured in a 96-well flat-bottom plate at concentration of 5×10^5 cells/ml. After 12 h of preconditioning, the cells were treated with various concentrations of BL for 24 h. Thereafter, culture medium was aspirated and 100 μ l of MTT dye (1 mg/ml in PBS: phosphate-buffered saline) was added; the cultures were incubated for 4 h at 37°C. The formazan crystals produced through dye reduction by viable cells were dissolved using acidified isopropanol (0.1 N HCl). Index of cell viability was calculated by measuring the optical density of color produced by MTT dye reduction at 570 nm.

NAD⁺-to-NADH Ratio Assay

The NAD⁺-to-NADH ratio was measured from whole-cell extracts of ECV304 endothelial cells using the Biovision NAD/NADH quantization kit (BioVision, Mountain View, CA, USA), performed according to the manufacturer's instructions.

HO Activity Assay

HO activity was determined at the end of each treatment. Briefly, harvested cells were subjected to three cycles of freeze-thawing before addition

to a reaction mixture consisting of phosphate buffer (1 ml final volume, pH 7.4) containing magnesium chloride (2 mM), NADPH (0.8 mM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 Units), rat liver cytosol as a source of biliverdin reductase, and the substrate hemin (20 μ M). The reaction mixture was incubated in the dark at 37°C for 1 h and was terminated by the addition of 1 ml of chloroform. After being vigorously vortexed and centrifuged, the extracted bilirubin in the chloroform layer was measured by the difference in absorbance between 464 and 530 nm ($\epsilon = 40 \text{ mM}^{-1}\text{cm}^{-1}$). The absorbance values recorded for each sample were normalized to mg of protein and the values were presented as the percentage of the control value.

Western Blot Analysis

Cells were washed with ice-cold PBS, scraped into PBS, and collected by centrifugation. Pellets were re-suspended in a lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA (ethylene glycol tetraacetic acid), 10% glycerol, 0.5% nonyl phenoxy polyethoxyl ethanol-40 (NP-40), 0.5% Tween 20, 1 mM dithiothreitol, and protease inhibitor cocktail and vortexed for 20 min at 4°C; insoluble material was removed by centrifugation. Protein (30 mg) was separated by SDS-PAGE. After transfer to nitrocellulose membrane, blots were blocked with PBS and nonfat milk (5%) and then incubated with antibodies directed against HO-1 (1:500), AMPK (1:500), p-AMPK (1:100), or b-actin (1:1,000). Membranes were washed in PBS, incubated with horseradish peroxidase-conjugated goat anti-rabbit, rabbit anti-mouse, or donkey anti-goat antibodies, and developed with commercial chemoluminescence reagents (Amersham, Arlington Heights, IL, USA).

Annexin V Binding Assay

Cells were stained with FITC-labeled annexin V (Genzyme Co., Cambridge, MA, USA) and PI for 30 min on ice. FITC-conjugated murine IgG monoclonal antibodies of unrelated specificity were always used as controls. After staining, the cells were washed and analyzed by FACS Vantage flow cytometry (Becton Dickinson; San Jose, CA, USA).

Statistical Analysis

Data are expressed as the means \pm SD. Statistical differences between groups were evaluated with Student's two-tailed *t*-test or by ANOVA

with Bonferroni's *t*-test when multiple groups were compared. Differences were considered to be significant when $p < 0.05$.

Results

Effects of BL and NAC Against TNF- α -Induced Cytotoxicity

ECV304 endothelial cells were treated with different concentrations of BL, and cell viability was performed after 24 h incubation. As shown in Figure 1A, no cytotoxic sign was observed up to 4 mM. However, the cell viability was significantly reduced at concentrations above 10 mM under our experimental conditions. Therefore, maximum concentration was limited to 4 mM of BL in all subsequent experiments. As previously reported²¹, incubation of TNF- α (40 ng/mL) for 24 h markedly reduced the viability of endothelial cells (Figure 1B). Interestingly, pretreatment with BL at 2 mM and 4 mM (but not 1 mM) for 6 h significantly attenuated TNF- α -induced cell death (Figure 1B). Cytoprotection comparable to that of BL at 4 mM was also observed when the cells were pretreated for 6 h with 10 mM of the antioxidant N-acetylcysteine (NAC) prior to TNF- α treatment (Figure 1B). It should be noted that the cytoprotective effect of BL was much higher when endothelial cells were pretreated with BL for 6 h prior to TNF- α treatment than when BL was added simultaneously with TNF- α (not shown).

Effects of BL on NQO1 and AMPK Activation

In ECV304 endothelial cells, BL increased the cellular NAD⁺-to-NADH ratio, which is associated with NQO1 activation, and this was significantly inhibited by DC, a specific inhibitor of NQO1 (Figure 2A). Next, we evaluated the effect of BL on AMPK activation, which is directly linked with its phosphorylation state. Endothelial cells were treated with BL at different concentrations (1, 2 and 4 mM) for 3 h, and the levels of AMPK phosphorylation were examined using Western blot analysis. An increase in AMPK phosphorylation was detected with BL at 2 mM (but not 1 mM) and BL at 4 mM showed a significant increase in AMPK phosphorylation, which was comparable to that of AICAR, an AMPK activator (Figure 2B). Interestingly, inhibition of NQO1 using DC abrogated the BL-induced phosphorylation of AMPK (Figure 2B). DC or AICAR alone had no significant effect on cell viability (not shown).

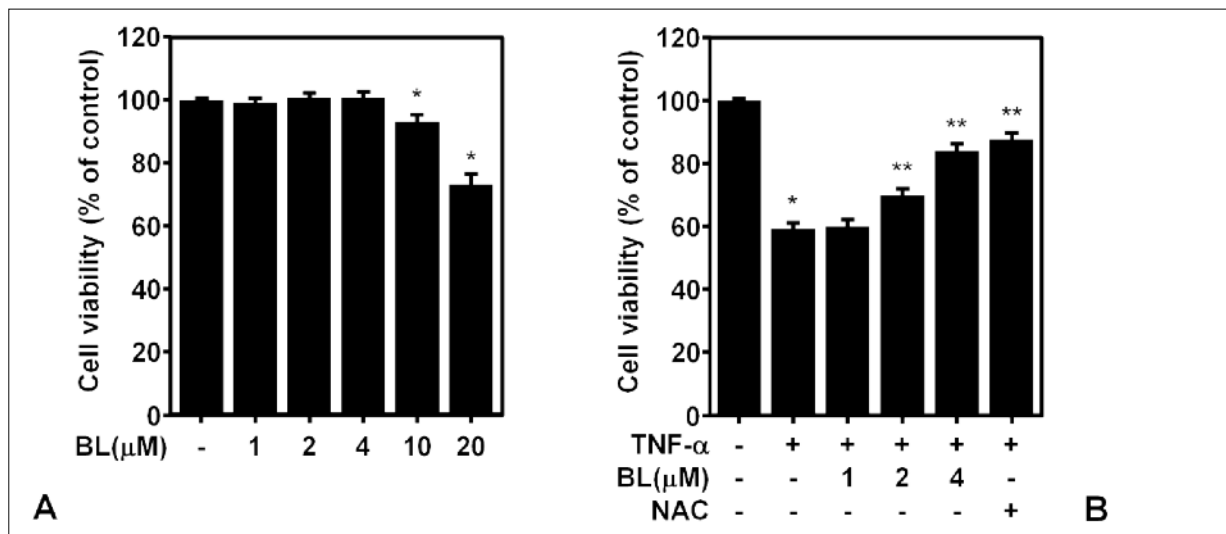


Figure 1. Effects of BL and NAC against TNF- α -induced cytotoxicity. **A**, ECV304 endothelial cells were treated for 24 h with indicated concentrations of BL. **B**, ECV304 endothelial cells pre-incubated for 6 h with indicated concentration of BL or 10 mM of NAC, and then exposed to 40 ng/ml of TNF- α for 24 h. Cell viability was measured as described under Materials and methods. Data are expressed as means \pm SD from 3 to 4 experiments. * p < 0.05 versus untreated group (control); ** p < 0.05 versus group treated with TNF- α alone.

Effects of BL on HO-1 Expression and HO Activity

In order to investigate the effect of BL on HO-1 expression, we treated ECV304 endothelial cells with BL at different concentrations (1, 2 and 4 mM) for 6 h (Figure 3A), and examined the levels of HO-1 expression. An increase in HO-1 ex-

pression was detected with BL at 2 mM, and BL at 4 mM showed a further increase in HO-1 expression (Figure 3A). To explore whether the AMPK pathway could be required for BL-induced HO-1 expression in ECV304 endothelial cells, we used CC, a specific inhibitor of AMPK, to inhibit AMPK activation, as well as DC that,

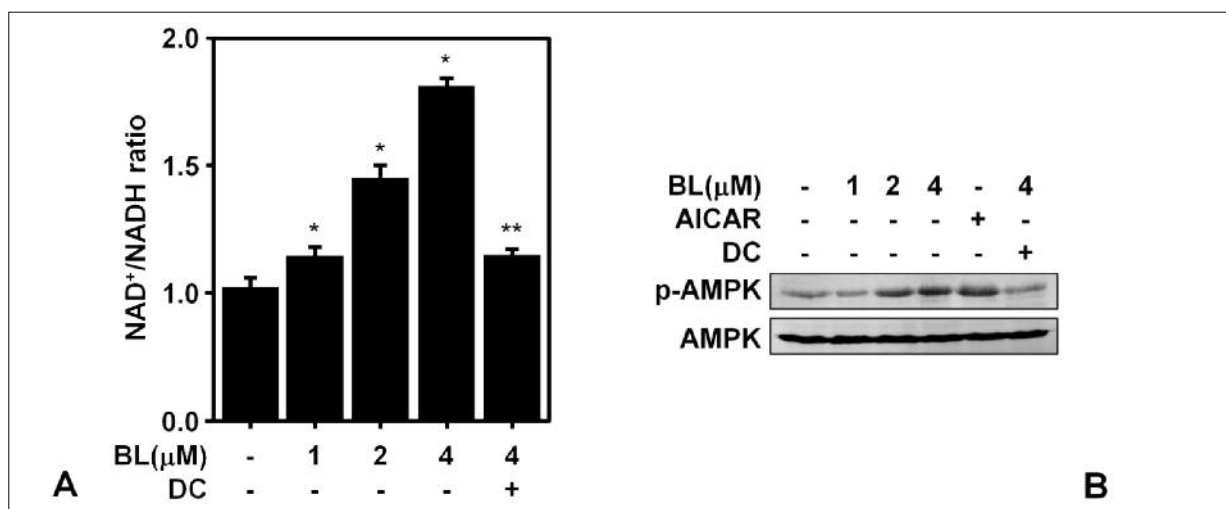


Figure 2. Effects of BL on NQO1 and AMPK activation. **A**, ECV304 endothelial cells were incubated for 6 h with indicated concentration of BL in the absence or presence of 10 μ M of DC; DC blocked BL-induced NADH oxidation. NAD⁺-to-NADH ratio assay was carried out as described under Materials and methods. Data are expressed as means \pm SE from 3 and 4 experiments. * p < 0.05 versus untreated group (control); ** p < 0.05 versus group treated with 4 μ M of BL alone. **B**, ECV304 endothelial cells were incubated for 6 h with indicated concentration of BL or 1 mM of AICAR in the absence or presence of 10 μ M of DC; DC blocked BL-induced AMPK phosphorylation. Western blot analysis for AMPK phosphorylation was performed as described under Materials and methods. Blots shown are representative of three independent experiments.

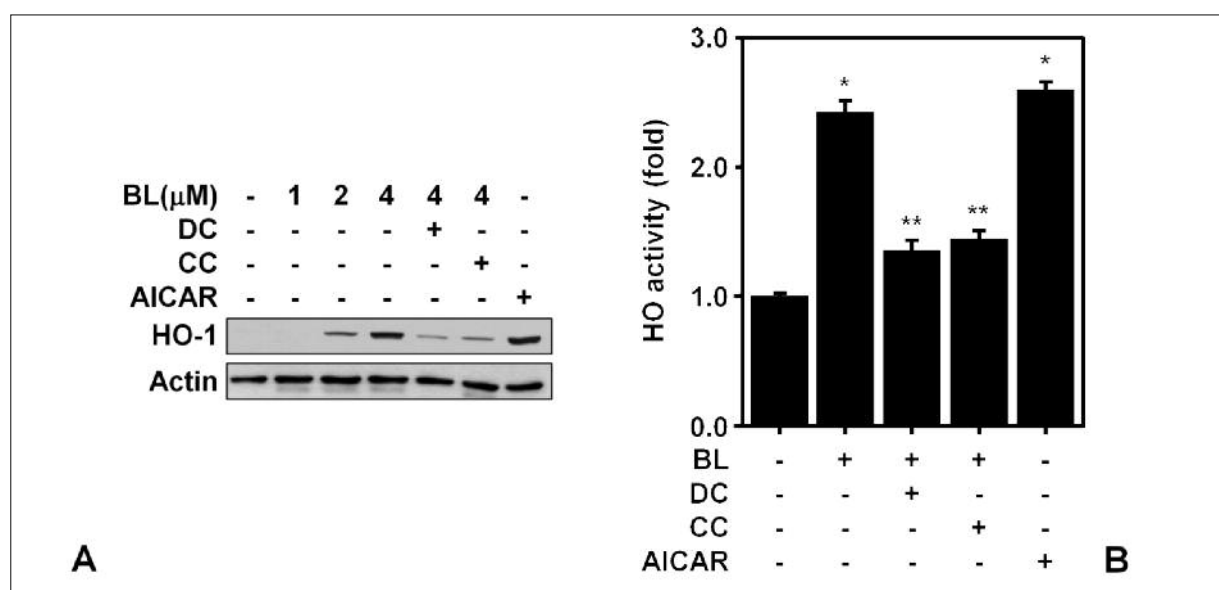


Figure 3. Effects of BL on HO-1 expression and HO activity. **A**, ECV304 endothelial cells were incubated for 6 h with indicated concentration of BL or 1 mM of AICAR in the absence or presence of 10 μ M of DC or 10 μ M of CC; DC and CC inhibited HO-1 expression. Western blot analysis for HO-1 expression was performed as described under Materials and methods. Blots shown are representative of three independent experiments. **B**, ECV304 endothelial cells were incubated for 6 h with 4 μ M of BL or 1 mM of AICAR in the absence or presence of 10 μ M of DC or 10 μ M of CC; DC and CC blocked HO activation. HO activity was measured as described under Materials and methods. Data are expressed as means \pm SE from 3 and 4 experiments. * p < 0.05 versus untreated group (control); ** p < 0.05 versus group treated with BL alone.

under our experimental conditions, was found to inhibit AMPK activation by selectively blocking NQO1 activity. Both CC and DC reversed BL-induced HO-1 expression in endothelial cells (Figure 3A). Conversely, AMPK activation by AICAR, an AMPK activator, induced HO-1 expression (Figure 3A). HO-1 expression by BL was associated with a marked increase in HO activity, and this was abolished by either CC or DC (Figure 3B). CC had no significant effect on cell viability (data not shown).

Cytoprotective Effects of AMPK Activation and HO-1 Expression

The observed cytoprotection by BL against TNF- α -mediated cytotoxicity was completely abolished in the presence of DC or CC, agents that indirectly or directly inhibit AMPK activation, and partly in the presence of the HO-1 inhibitor SnPP (tin protoporphyrin-IX) (Figure 4A). A cytoprotective effect comparable to BL was also observed when ECV304 endothelial cells were pretreated with the AMPK activator AICAR (Figure 4B). Whereas CC completely abolished AICAR-induced cytoprotection, only a partial attenuation was observed in the presence of SnPP (Figure 4B). As shown in Figure 4B, DC

was without influence on AICAR-induced cytoprotection, because this agent has only an inhibitory effect on NQO1 activation (but not on AMPK activation).

Finally, TNF- α -mediated apoptosis in ECV304 endothelial cells was quantified by flow cytometry after annexin V and PI staining (Figure 5A). As shown in Figure 5B, TNF- α increased the cell population that was positive for annexin V, which was significantly decreased by BL treatment. A substantial number of the annexin V-positive cells were not stained by PI, indicating that they are apoptotic (not necrotic) under this specific experimental condition (Figure 5A). Anti-apoptotic effect of BL was completely abolished by DC or CC, and partly by SnPP (Figure 5B). It was worth noting that the cytoprotective results obtained from MTT assay were similar to those obtained by flow cytometry.

Discussion

The present study demonstrates that the naturally occurring NQO1 substrate BL is capable of protecting ECV304 cells, which are derived from normal human vascular endothelial cells, from

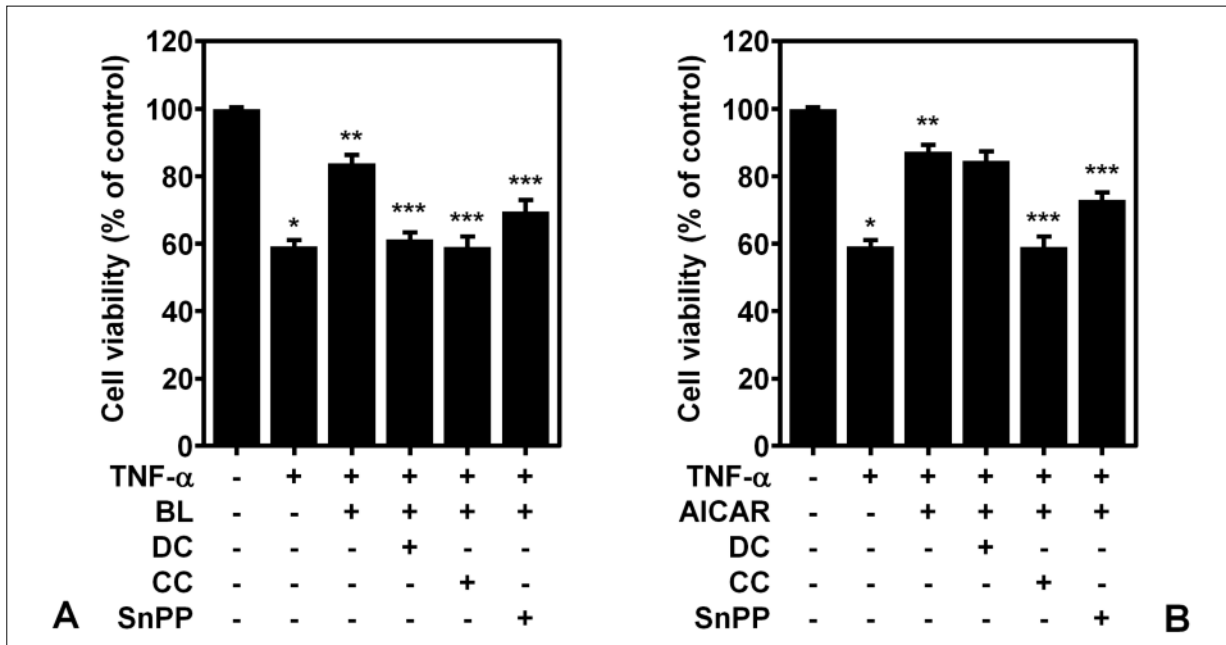


Figure 4. Cytoprotective effects of BL-induced AMPK activation and HO-1 expression. **A**, ECV304 endothelial cells pre-incubated for 6 h with 4 μ M of BL in the absence or presence of 10 μ M of DC, 10 μ M of CC or 10 μ M of SnPP were exposed to 40 ng/ml of TNF- α for 24 h. **B**, ECV304 endothelial cells pre-incubated for 6 h with 1 mM of AICAR in the absence or presence of 10 μ M of DC, 10 μ M of CC or 10 μ M of SnPP were exposed to 40 ng/ml of TNF- α for 24 h. Cell viability was measured as described under Materials and methods. Data are expressed as means \pm SD from 3 to 4 experiments. * p < 0.05 versus untreated group (control); ** p < 0.05 versus group treated with TNF- α alone; *** p < 0.05 versus group treated with BL or AICAR plus TNF- α .

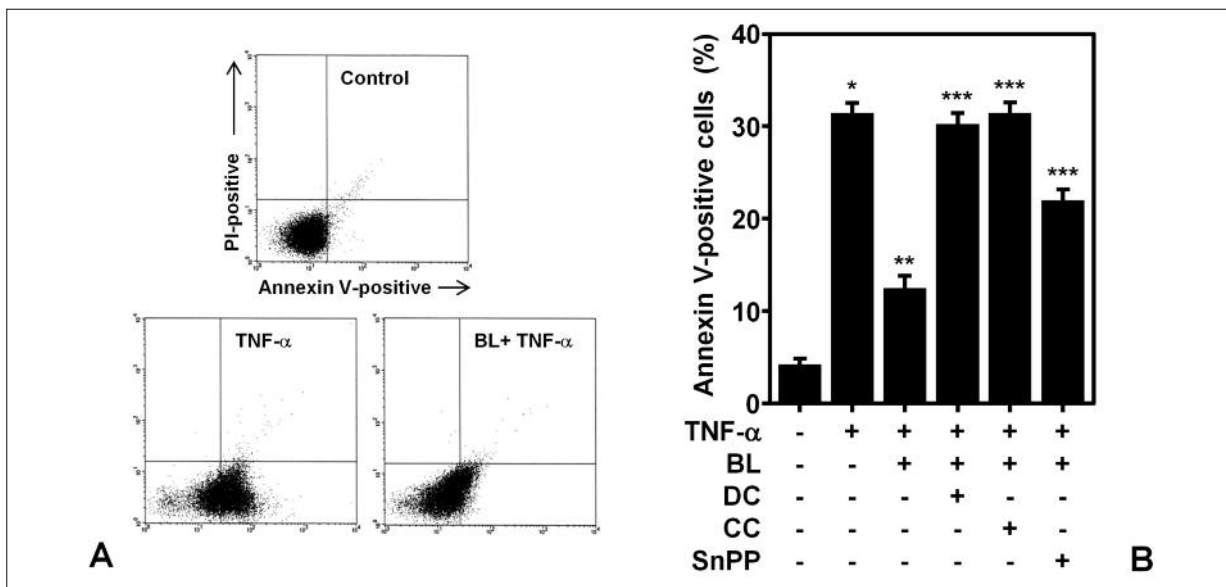


Figure 5. Anti-apoptotic effects of BL-induced AMPK activation and HO-1 expression. **A**, ECV304 endothelial cells were incubated for 6 h with or without 4 μ M of BL, and then exposed to 40 ng/ml of TNF- α for 12 h. Apoptotic cells were quantified by a flow cytometry using Annexin V-FITC and PI, as described under Materials and methods. Histograms shown are representative of three independent experiments. **B**, ECV304 endothelial cells pre-incubated for 6 h with 4 μ M of BL in the absence or presence of 10 μ M of DC, 10 μ M of CC or 10 μ M of SnPP were exposed to 40 ng/ml of TNF- α for 24 h. Data are expressed as means \pm SD from 3 to 4 experiments. * p < 0.05 versus untreated group (control); ** p < 0.05 versus group treated with TNF- α alone; *** p < 0.05 versus group treated with BL plus TNF- α .

cytotoxicity caused by the circulatory pro-inflammatory cytokine TNF- α . Other studies have reported the direct cytotoxic effects of BL in several types of tumor cells, although at micromolar concentrations²²⁻²⁵. In line with these studies, BL indeed reduced cell viability only at the concentrations above 10 mM under our experimental conditions. However, BL appeared to be predominantly cytoprotective at the lower concentrations. We also demonstrate that BL seems to exert a cytoprotective effect, at least under our experimental conditions, by inducing AMPK activation and subsequent expression of the cytoprotective HO-1.

Plasma levels of TNF- α are increased in diabetes. In particular, the levels of TNF- α seem to increase in parallel with the severity of diabetes and oxidative stress²⁶. TNF- α has been shown to both be secreted by endothelial cells and to induce intracellular ROS formation²⁷. These observations provide a potential mechanism by which TNF- α may activate and injure endothelial cells, subsequently resulting in endothelial dysfunction. In our study, exposure of ECV304 endothelial cells to TNF- α induced cell death, and the well-known antioxidant NAC significantly attenuated TNF- α -induced endothelial cell death, implying an important role of ROS formation in endothelial cell death caused by TNF- α .

The enhancement of NADH oxidation by BL in NQO1-present cells has been shown to stimulate AMPK activation^{19,20}. In this study, we sought to investigate whether BL could also stimulate AMPK activation in ECV304 endothelial cells. BL increased NQO1-mediated NAD⁺-to-NADH ratio and AMPK phosphorylation. Similar phosphorylation of AMPK was also observed when the endothelial cells were exposed to the well-known AMPK activator AICAR. Moreover, the NQO1-specific inhibitor DC almost completely blocked BL-induced AMPK phosphorylation, suggesting that BL may induce AMPK activation specifically by NQO1 activation. These findings, therefore, indicate that BL is also capable of stimulating AMPK activation in ECV304 endothelial cells, perhaps by enhancing NQO1 activity. The increased intracellular levels of NAD⁺ have been reported to activate AMPK in skeletal muscle cells and cardiomyocytes^{28,29}, but the underlying mechanism(s) is not well understood yet. Because the intracellular NAD⁺-to-NADH ratio indicates the energy status of cells³⁰, the AMPK signaling pathway, a well-known energy sensing pathway, may be activated under

energy depletion. Thus, it is most likely that BL-induced NADH oxidation in endothelial cells may transiently activate AMPK to compensate for cellular energy depletion.

Although AMPK is traditionally thought of as an intracellular energy switch, recent evidence has shown that this molecule also plays important roles in maintaining physiological processes in the vasculature³¹. In the endothelium, AMPK appears to attenuate ROS-mediated stress associated with vascular pathology⁶. However, the mechanism(s) by which AMPK could have cytoprotective/antioxidant properties is not completely elucidated as yet. Interestingly, recent studies have demonstrated that AMPK activation has an ability to induce HO-1 expression that can exert cytoprotective effects^{8,9}. In our study we, therefore, tested the effect of AMPK activation by BL on HO-1 expression in ECV304 endothelial cells. BL increased HO-1 expression and subsequent HO activity. The expression of HO-1 by BL was dependent on AMPK activation, because both CC, a pharmacological inhibitor of AMPK activation, and DC, an inhibitor that blocked AMPK phosphorylation in our study, prevented an increase in HO-1 expression. In support of these findings, the AMPK activator AICAR was also capable of inducing HO-1 expression in the endothelial cells. Our results, therefore, indicate that BL can induce HO-1 expression through activation of AMPK in endothelial cells. The mechanism by which AMPK activation can induce HO-1 expression in endothelial cells has been well established by Liu et al⁸. They showed that treatment of endothelial cells with AICAR induced HO-1 expression that was associated with a prominent increase in nuclear factor-erythroid 2-related factor 2 (Nrf2), indicating that AMPK induces HO-1 expression *via* Nrf2 pathway.

HO-1 expression has been shown to exert significant cytoprotective effects in a variety of cell types, including endothelial cells¹¹⁻¹⁴. In light with this, we explored the potential involvement of HO-1 in cytoprotection by BL in ECV304 endothelial cells. BL markedly increased HO-1 expression by activating AMPK, and the expression correlated with the decrease in TNF- α -induced cell death. Inhibition of HO-1 activity by SnPP partly and not completely abolished the cytoprotective effect of BL, suggesting that the cytoprotective effect of BL against TNF- α is associated, at least in part, with HO-1 expression. Interestingly, inhibition of NQO1 and AMPK by DC and CC, respectively, almost completely abolished

cytoprotection by BL. Thus, it is reasonable to assume that BL-induced AMPK activation, together with HO-1 pathway, might also activate other cytoprotective pathways and overall cytoprotective effects of BL could be achieved by virtue of the concerted actions of the multiple pathways being activated. This assumption is also supported by our findings demonstrating that the observed cytoprotection by AICAR was completely abolished by CC, whereas partly by SnPP. The beneficial effects of HO-1 expression have been attributed to several factors, including the degradation of pro-oxidant heme, formation of biliverdin and/or bilirubin with their antioxidant properties, as well as the release of carbon monoxide, which has cytoprotective and anti-inflammatory effects¹⁰. Although the exact mechanisms involved in cytoprotective/antioxidant actions of the HO-1 system have not been fully elucidated, one or more of the HO-1 reaction products may mediate the cytoprotective effects of BL-induced AMPK activation under our experimental conditions.

Extracellular matrix degradation is important in the pathogenesis of atherosclerosis. At early stages of atherogenesis, basement membrane components can be degraded by released proteolytic enzymes, including the matrix metalloproteinases (MMPs) and serine proteases³². Among the MMPs, MMP-9, when induced aberrantly or inordinately, can injure tissues, and is incriminated in the pathogenesis of autoimmune diseases and atherosclerosis³³. By disrupting basement membranes, MMP-9 facilitates proliferation and migration of smooth-muscle cells, and the influx of inflammatory cells, processes fundamental to the pathogenesis of neointimal hyperplasia and atherosclerosis, and destabilization of atherosclerotic plaques³³. In endothelial cells, MMP-9 is inducible by oxidants and pro-inflammatory cytokines. Indeed, it has been reported that MMP-9 expression is induced by ECV304 endothelial cells stimulated with non-cytotoxic TNF- α ³⁴. Interestingly, there was increased marked induction of MMP-9 in HO-1-knockout mice³⁵, suggesting that endothelial HO-1 expression may reduce

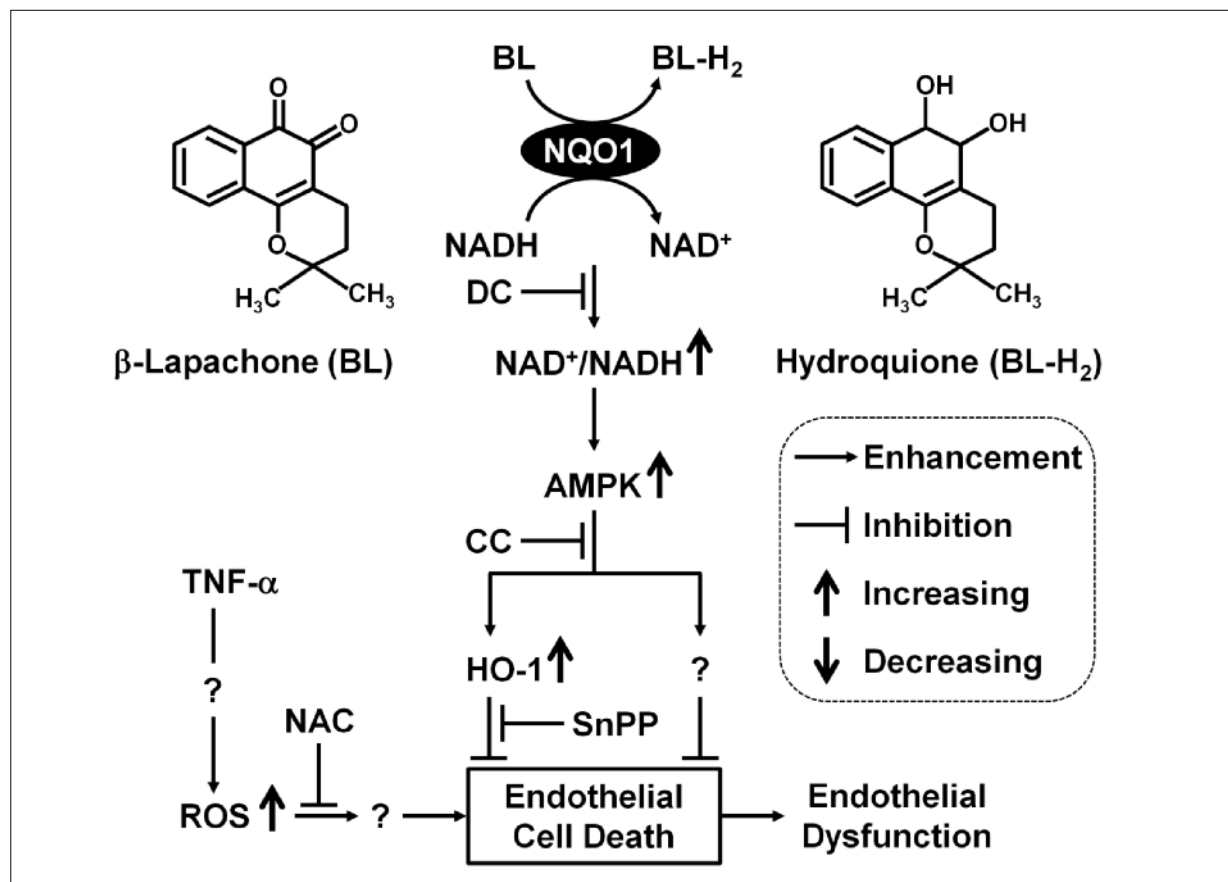


Figure 6. Schematic model for the cytoprotective mechanism of BL in TNF- α -induced endothelial cell death.

MMP-9 expression and secretion. Thus, it is most likely that HO-1 expression by BL may inhibit MMP-9 expression and secretion in TNF- α -stimulated ECV304 endothelial cells. However, further studies are needed to determine if BL could reduce TNF- α -induced expression of MMP-9 and other MMPs *via* HO-1 expression in ECV304 endothelial cells. Recently, it has been reported that endothelium-selective activation of AMPK prevents diabetes mellitus-induced impairment in vascular function and re-endothelialization *via* induction of HO-1 in mice³⁶. In this regard, it is most likely that BL may have therapeutic potential to interfere with the progression of atherosclerosis and its complications, because of its ability to induce AMPK activation and HO-1 expression in human endothelial cells.

Conclusions

The present study demonstrates that BL can induce endothelial HO-1 expression in ECV304 cells by enhancing AMPK activation *via* NADH oxidation by increased NQO1 activity. This study also demonstrates that AMPK activation and subsequent HO-1 expression confer cytoprotection against TNF- α -induced endothelial cell death, as briefly summarized in Figure 6.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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